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14. ABSTRACT
Fibroblast growth factors (FGFs) are vital modulators of development as well as angiogenesis. FGFs play a large role in vascular formation, body axis patterning, cell migration and organ branching. The scientific community is still piecing together the role that distinct FGFs play due to the complexity of the FGF network, which involves 22 distinct members that signal through 4 receptors to activate 3 major signaling pathways. As an added layer of complexity, FGF binding proteins (FGFBPs) release and activate FGFs from the extracellular matrix. FGFs act as major angiogenic factors and have therefore been of interest for therapeutic targeting. Success may rely on further elucidation of the regulation involved. The redundancy of FGF has made current FGF targeted therapies only moderately effective. Overexpression of human FGFBP1 in a conditional mouse model leads to decreased tertiary mammary ductal branching caused by increased epithelial apoptosis. This phenotype is seen only in mature mice that have fully developed mammary glands as opposed to pubertal mice developing altered mammary glands. FGFs have been strongly implicated in both dorsal/ventral axis patterning as well as mammary gland branching. This work supports the hypothesis that overexpressing human FGFBP1 in a developed mammary gland results in altered mammary gland structure. Furthermore, expression of human FGFBP1 in mammary tumors results in increased tumor size possibly due to FGFBP acting in its role as an angiogenic switch.

15. SUBJECT TERMS
Fibroblast Growth Factor Binding Protein-1, mammary gland development, reduced tertiary branching, apoptosis

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INTRODUCTION

A secreted fibroblast growth factor-binding protein (FGFBP1) can enhance the activity of locally stored, immobilized FGFs in the extracellular membrane. Fibroblast growth factors (FGFs) act as potent mitogens during embryogenesis, cell differentiation, and proliferation. Through its function as an angiogenic switch, we hypothesize that FGFBP1 can support tumor growth through facilitation of new vessels to feed the growing tumor and through inducement of cell proliferation. To further study the role that this protein has on tumorigenesis, transgenic mice were generated containing human FGFBP1 under a tetracycline inducible transgenic mouse system. Preliminary data indicate a striking decrease in the lateral budding of mammary glands in animals expressing human FGFBP1 (hFGFBP1). Matrigel plug assays, wound healing studies and ischemic models in the transgenic mice showed increased angiogenesis, fibroblast and keratinocyte proliferation and macrophage invasion. Drastic changes were seen in arterial blood pressure as soon as 24 hours after the hFGFBP1 gene was induced indicating a role in vessel regulation and maintenance. Furthermore, other studies have shown FGFBP1 is upregulated in the progression from normal to *in situ* carcinoma of the breast as well as in further progression to invasive breast cancer in patients. Normally down regulated in adults, FGFBP1 is seen at high levels in cell lines derived from squamous cell carcinomas and some colon cancers as well as xenografted squamous cell carcinomas. Other studies have indicated that FGFBP1 is the second most predominant protein purified from bovine mammary secretions on a heparin column during the last trimester of gestation. FGF-2 signaling has been shown to be an important signal pathway in pregnancy dependent lobuloalveolar development in the mammary gland. This data suggests that FGFBP1 has an important role in the biology of the mammary gland and may play an important role in mammary tumorigenesis.

Evaluation of expression in established cell lines by Northern blot showed that FGFBP1 was found in several carcinoma cell lines, including squamous cell carcinoma from bladder, lung and cervix. Breast and prostate lines have also shown high levels of FGFBP1 expression. Only keratinocytes showed expression in normal cell lines tested for BP expression. In tissue samples, FGFBP1 protein and mRNA are expressed at increasing frequencies and levels during disease progression including: malignant progression of colon cancer, pancreatic duct epithelia to *in situ* lesions, and several other invasive cancers (Aigner et al., 2001; Ray et al., 2006; Ray et al., 2003; Tassi et al., 2001; Tassi et al., 2006; Tassi and Wellstein, 2006). BP1 potentially contributes to the increasing angiogenic phenotype during this progression (Czubayko et al., 1997).

The embryonic expression of the endogenous mouse homologue of FGFBP1 has been evaluated extensively. *In situ* hybridization has indicated that mFGFBP1 is expressed in mouse embryonic skin, intestine, and lung but is down regulated postnatally (Kurtz et al., 1997). Immunohistochemistry (IHC) reveals protein upregulation of FGFBP1in the skin, olfactory epithelium, tracheal epithelium, primitive tubuli and to a slightly lesser extent, primitive glomeruli, and thymus. Sampling of different mouse embryonic stages demonstrated that expression levels alter during different embryonic stages (Aigner et al., 2002). There is a correlation between FGF2 localization and mFGFBP1 localization in embryonic development of the mouse with the one major difference being that while FGFBP1 is seen in the epithelium, FGF2 is largely associated with the extracellular matrix (ECM) (Gonzalez et al., 1990). This model supports the idea of FGFBP1 as a secreted protein that releases FGF from the ECM in an autocrine or paracrine manner. FGFBP1 is thought to bind FGFs after release from the cell. FGFBP1 binding extricates FGFs from the ECM by decreasing the affinity of FGFs for heparan sulfates. The low affinity binding of FGFBP to FGFs allows FGFBP to present FGFs to high affinity tyrosine kinase receptors (FGFR) on the cell surface.

As the only branched organ that undergoes the majority of its development during adolescence and adulthood rather that during the embryonic state, the mammary gland offers a fascinating opportunity to study organ formation without trying to bypass embryonic development issues. Mammary development, including branching, is largely directed by hormonal and growth factor signaling. While the hormonal interactions have been extensively investigated, effects due to growth factor signaling, specifically FGFs, have yet to be

completely elucidated. To that end, we have used a conditional transgenic mouse model that expresses hFGFBP1 and evaluated the effects that modulation of FGF signaling has on murine mammary glands. To evaluate the effects of FGFBP1 expression on mammary tumorigenesis, we utilized several established mammary tumor models including the Her2/neu mice and the PyMT(FVB) mice.

BRIEF SUMMARY OF NORMAL STAGING IN MURINE MAMMARY DEVELOPMENT

1. Embryonic

Mouse mammary gland development begins after mid-gestation with the formation of milk lines, which consist of two bilateral epidermal ridges that run from the hindlimb up to the forelimb on both sides of the embryo (Veltmaat et al. 2003; Hens and Wysolmerski 2005). At the future site of each nipple, five disk-like placodes line up and invade into the underlying mesenchyme. Referred to as the anlage, this bud enters a knot of preadipocytes that are destined to become what is considered the mammary fat pad. The anlage then branches more than 10 times to form a rudimentary ductal tree (Hinck and Silberstein 2005). There are multiple signaling pathways implicated in this process. Those that are required for normal embryonic gland formation include the Wnt pathway (Andl et al. 2002) the FGF signaling pathway (Mailleux et al. 2002) and parathyroid related hormone (Wysolmerski et al. 1998). Here we have focused on the effects that FGF may have on the mammary gland and know regulation by sex hormones. Signaling pathways that are not required for embryonic gland development include the estrogen receptor (Couse and Korach 1999) and the progesterone receptor (Hovey et al. 2002) as shown by knockout models. Unlike the human breast, which forms several trees, the mouse mammary gland forms a single ductal tree leading to each nipple. The nascent mammary gland remains quiescent until puberty where the introduction of sex hormones (estradiol/progesterone) and the resulting growth factor signaling induces explosive growth and differentiation (Howard and Gusterson 2000).

2. Adolescence/Puberty

The ovarian secretion of estrogen and progesterone occurs in response to a rise in the level of gonadotrophins during this stage. The result in the mammary gland is a rapid invasion of ductal epithelium into the surrounding stromal fat pad. This phase generally begins when the mouse is 4-6 weeks and ends around 12 weeks of age (Howlin et al. 2006). Terminal end buds (TEBs) form at the periphery of the immature ducts and are the leading edge of the penetrating ducts. As the ducts elongate into the fat pad, the majority of cellular proliferation takes place at the tip of the TEB. Elongation continues until the entire fat pad has been overrun. New primary ducts are formed by bifurcation of the TEB and secondary branches begin sprouting off perpendicularly from the primary ducts. (Hennighausen and Robinson 1998; Hennighausen and Robinson 2001; Sternlicht et al. 2006)

Studies have shown that although the nascent mammary gland is refractory to estrogen/progesterone treatment, mammary gland growth and elongation of epithelial ducts is stimulated and regulated by estradiol and the estrogen receptor α (ERα) (Fendrick et al. 1998). By disrupting the ERα in a mouse model, it was shown that estrogen receptor α is required for ductal elongation during puberty (Korach et al. 1996; Bocchinfuso and Korach 1997) Further studies defined the requirement for ERα in the stromal compartment during ductal growth. Mueller et al showed that stromal ERα was necessary regardless of the epithelial expression by transplanting wild type epithelial cells into ERα knockout stroma. However, ERα deficient mammary epithelial cells were unable to develop epithelial ductal structures in an ERα positive stroma, indicating that ERα is also required in the epithelium. Interestingly, the decrease in mammary duct elongation due to ERα knockout could be rescued by the treatment of high doses of estradiol and progesterone(Cunha et al. 1997; Mueller et al. 2002).

3. Adult Maturation

As the mammary gland is subjected to repeated cycles of ovarian stimulation, the ductal tree is filled out completely. During this stage, elongated ducts form lateral branches or buds, which are distinct from TEB

bifurcation. Lateral branches form at separate sites along the ducts and represent controlled sprouting of the epithelium into the surrounding fat pad. They are also referred to as tertiary branches or side branches with end buds or alveolar sprouts (Robinson et al. 1999; Brisken 2002; Hennighausen and Robinson 2005; Lu et al. 2006). This strategy of using a branched system to acquire a large epithelial surface in a limited tissue volume is seen in multiple organs and organisms and represents an evolutionarily conserved process.

Functional deletion of progesterone receptor (PR) led to defects in side branching in virgin mice (Humphreys et al. 1997). Further transplant studies showed prevention of normal lobuloalveolar development and the formation of tertiary branching in virgin mice where PR was absent in transplanted donor epithelium. Defects in development were not seen when PR was absent in recipient stroma indicating that PR is necessary in the epithelium but not the stroma (Brisken et al. 1998). Atwood et al. showed that administration of progesterone (by pellet) does indeed induce increased side branching normal mice. Furthermore, normal side branching corresponds with an increase in progesterone serum levels (Atwood et al. 2000). This data supports earlier studies indicating that progesterone stimulates branching (Haslam 1988b; Haslam 1988a). As noted earlier, other factors play a role in mammary gland branching and it should be noted that Humphreys et al. claimed that there was a possible role for a secondary but not yet identified growth factor signal in conjunction with progesterone signaling (Humphreys et al. 1997).

Other signaling during branching involves prolactin, which has been shown to act directly on mammary epithelium to induce alveolar development but acts through an indirect mechanism to influence ductal branching (Brisken et al. 1999; Ling et al. 2000). This is likely tied to prolactin's ability to stimulate synthesis and secretion of progesterone (Bole-Feysot et al. 1998). Although estrogen is not directly associated with side branching, it indirectly affects ductal development by elevating both prolactin and progesterone levels and inducing progesterone receptors in mammary epithelium (Edery et al. 1985; Imagawa et al. 1985; Bocchinfuso et al. 2000)

4. Pregnancy/Lactation/ Involution

This stage involves massive tissue remodeling with rapid and global proliferation of epithelial cells within the ductal branches and alveoli (Richert et al., 2000). The single epithelial cell layer of the alveoli is surrounded by a discontinuous layer of myo-epithelial cells capable of contraction. Contact with the ECM is required for complete differentiation of the epithelial cells (Fata et al., 2004). As the mouse nears parturition, tight junctions in the alveoli close and milk and cololstrum proteins move into the alveolar lumen in preparation for milk secretion. Along with the epithelial expansion, adipocytes lose their lipid content and the vasculature remodeling occurs to provide the necessary energy, sugars, amino acids etc needed for milk production (Neville et al., 1998). This cycle requiring explosive epithelial expansion after which massive programmed cell death occurs indicates the existence of persistent self renewing mammary stem cells capable of expanding into separate lineages necessary for mammary epithelial maintenance and milk production (Smalley and Ashworth, 2003; Smalley and Clarke, 2005; Stingl et al., 2005).

BODY

Task 1 from the original statement of work was to determine the role of fibroblast growth factor binding protein 1 (FGFBP1) in mammary gland development. To do this, transgenic mice expressing human FGFBP1 were utilized. FGFBP1 will be referred to as either FGFBP or BP in this text. The conditional tet-off mice were evaluated for human FGFBP1 expression in the mammary gland by real time PCR (RT-PCR) and by immunohistochemistry (IHC) (Figure 1 and 2). When doxycycline is removed from the diet of these mice, the tet operon is able to produce hFGFBP1. All studies involving these animals achieve induction of hFGFBP1 by removal of doxycline from their diet unless otherwise noted.

1. **Validation of mouse model system.** Inguinal #4 mammary glands were harvested from transgenic mice on the doxycyline (doxy) diet, transgenic mice expressing the protein (FGFBP1) on regular diet and transgene negative littermates on both diets. Samples were fixed in formalin and embedded in paraffin. Using a human BP specific monoclonal antibody, immunohistochemistry (IHC) and using sequence specific primers RT-PCR was performed (Figure 1 and 2). Staining was evident in the ductal stroma in transgenic mice expressing hFGFBP1 (Figure 2). There was slight staining in transgenic mice on the doxy diet that is expected under the slightly leaky tet expression system. Negative controls showed no staining regardless of diet. RT-PCR showed significant increase in expression. (Figure 1.)

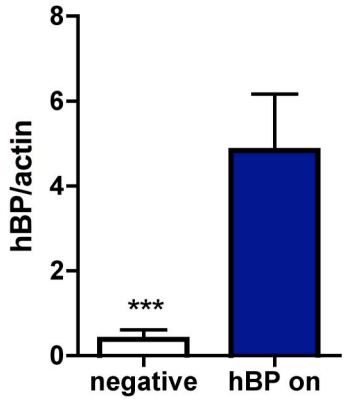


Figure 1. Transgenic mice express significant levels of hFGFBP1 in the mammary gland as shown by quantitative real time PCR. The RNA from #3 mammary gland was measured from transgenic mice 60 days old that were induced to express hFGFBP1 for 30 days. This RNA was compared to mice negative for the hFGFBP1 transgene as a negative control. This experiment was done with n=5 for both groups and samples being analyzed in duplicate. Expression levels were normalized for actin expression. *** Indicates p value < 0.01.

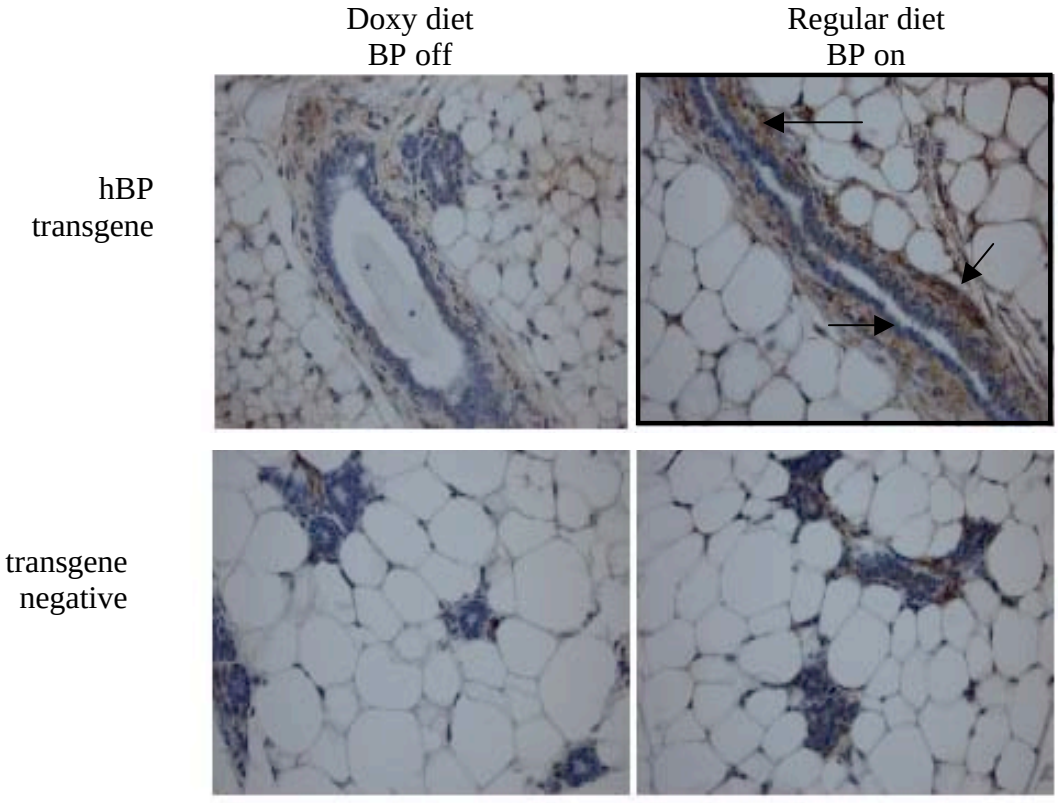


Figure 2. Expression of hFGFBP1 protein in transgenic mice as shown by immunohistochemistry. Virgin mice were induced to express hFGFBP1 by removal of doxycycline from their diet at 90 days of age and were sacrificed at 120 days. The 4th inguinal mammary gland was removed and formalin fixed for paraffin embedding. Positive staining is outlined by in black with arrows.

Task 1 continued. Effects of hBP1 on murine mammary gland development

1. Decreased budding phenotype of mammary glands in adult mice.

Mammary glands from mice that were chronically induced to express hFGFBP1 from 90 to 120 days of age were analyzed by whole mounting the 4th inguinal mammary gland. This time period involves a completely developed adult mammary gland as described in the introduction. While the controls showed no difference in branching, a significant difference was seen in adult animals expressing the hFGFBP1 transgene (Figure 3). There was a dramatic decrease in tertiary branching. Earlier time points with the same period of hFGFBP1 induction resulted in a few samples (not statistically significant) demonstrating the reduced branching but the majority were unaffected by transgene expression and may be due to animal developmental differences. (Figure 4.) Additional hFGFBP1 due to induction therefore seems to have an impact on the developed gland but does not impact the developing gland.

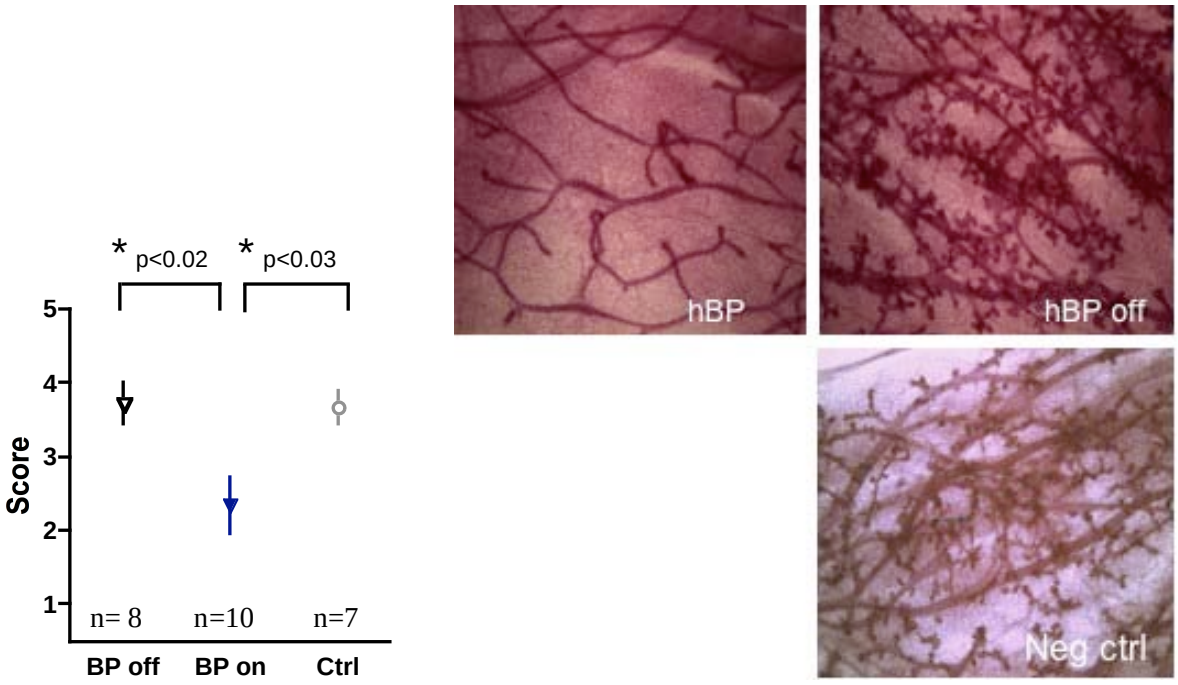


Figure 3. Chronic 30 day transgene induction of hFGFBP1 results in decreased mammary tertiary branching. Transgenic mice were induced to produce hFGFBP1 at 90 days of age and were sacrificed at 120 days. The inguinal #4 mammary gland was whole mounted and stained with Carmine Alum stain. Mammary glands were scored with 4 being the most tertiary branching and 1 being almost none.

2. Earlier Developmental time points resulted in no significant difference in branching.

To evaluate hFGFBP1 effect on developing branching during puberty, mice were induced to express hFGFBP1 at 30 days and were sacrificed at 60 days during the early puberty stage. The fourth inguinal mammary gland was harvest and stained with Carmine alum. Branching was analyzed as described previously. No significant difference was found between the hFGFBP1-on group and the hFGFBP1-off (Figure 4A). Scoring was similar

to the corresponding non-transgenic littermates suggesting that mice not expressing hFGFBP1 show a wild type phenotype. A second time point to evaluate branching during lateral bud formation as they cycle through multiple estrous cycles during the later pubertal stages, mice were induced at 60 days of age and sacrificed at 90 days. This time point showed results similar to the earlier time frame of 30-60 days (Figure 30B). No difference was seen in terminal end buds from induced mice as compared to wild type controls.

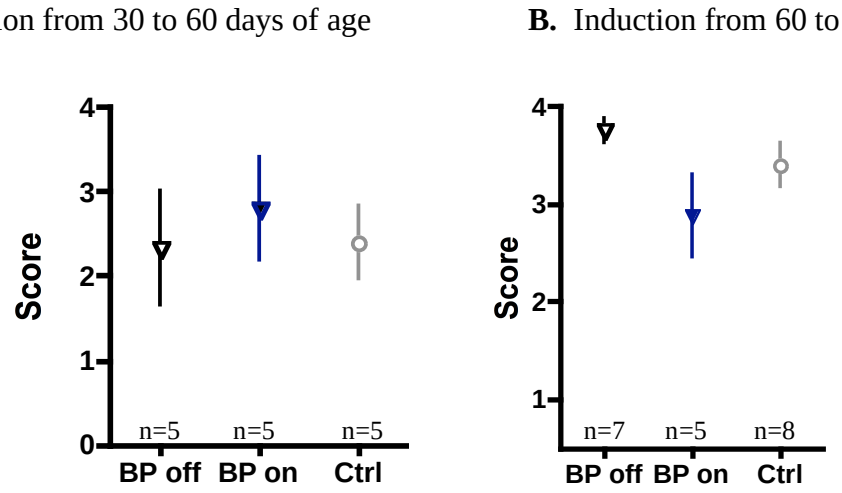
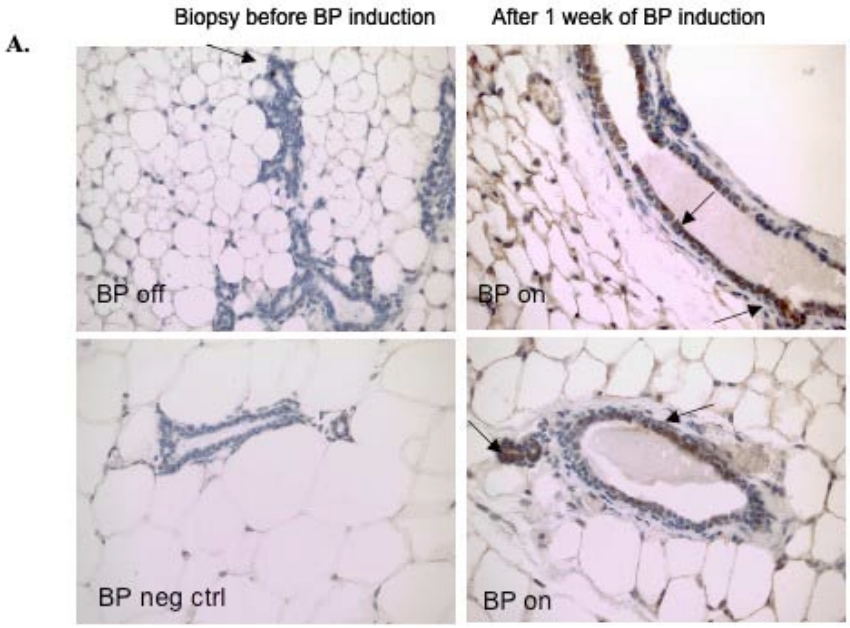


Figure 4. Chronic 30 day induction of hFGFBP1 does not impact branching in mammary glands that are not completely developed. **A.** Transgenic mice were induced to produce hFGFBP1 at 30 days and sacrificed at 60 days. The inguinal #4 mammary gland whole mount was scored and evaluated in a blind manner. There were several glands with diminished branching but the phenotype was seen in all groups including controls. **B.** Mice induced to produce hFGFBP1 by removal of doxy at 60 days and sacrificed at 90 days showed no significant difference in lateral budding or tertiary branching.



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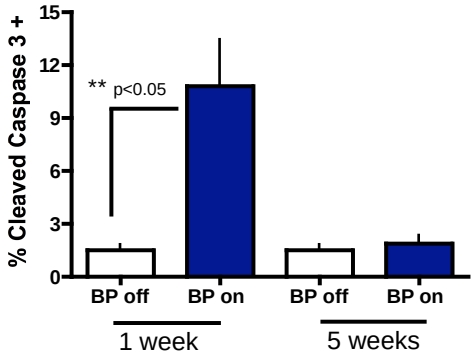

Figure 5. Apoptosis is increased after a 1week acute induction of hFGFBP1 in mammary glands from transgenic mice. Apoptotic levels return to background levels after a chronic induction of 5 weeks. Paraffin embedded samples were analyzed by IHC of cleaved caspase-3 and positive cells were counted. This experiment was performed with an n=4 for each group. **A.** Shows representative samples. Arrows show positive cells and **B.** Quantitation of cleaved caspase-3 positive cells.

Wild type murine mammary glands were analyzed for expression of mouse FGFBP1 (mFGFBP1) during various mammary developmental stages (Fig 6). Expression of mFGFBP1 was high in virgin adult mammary glands but levels dropped during pregnancy and went back up during involution. Our earlier data showing that in the tTA/tetBP mice, FGFBP1 plays a role in apoptosis would be supported by this expression pattern. As the mammary gland goes through dramatic proliferation stages preparing for lactation, FGFBP1 expression is lost and as the mammary gland goes through apoptotic remodeling, expression of FGFBP1 goes back up to earlier levels.

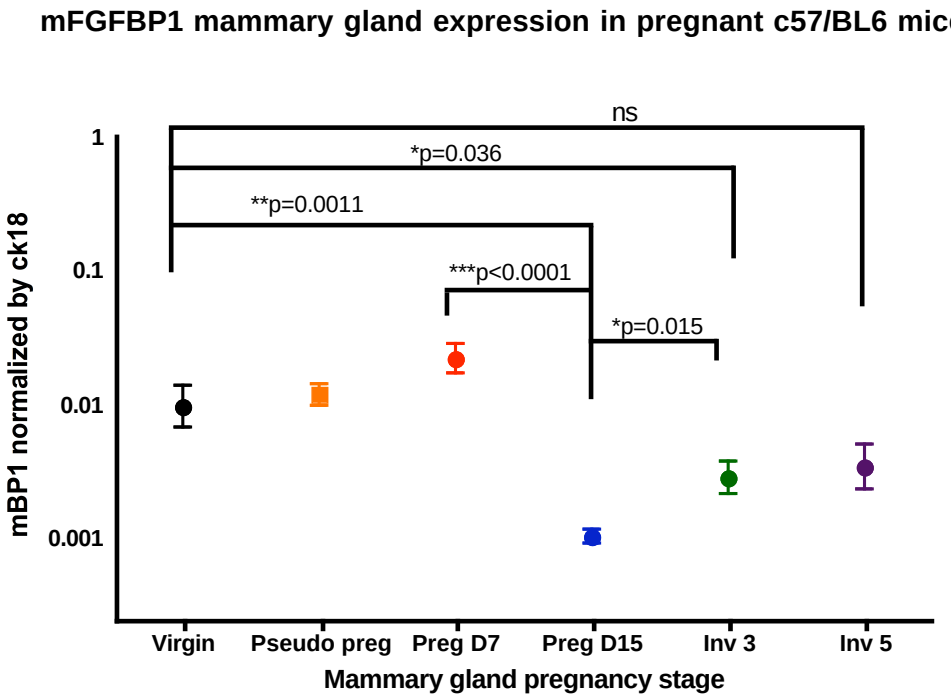

Figure 6. Virgin mammary glands show expression of mouse FGFBP1 but expression decreases during later stages of pregnancy and increases again during involution stages. Mammary glands were harvested at stages indicated and RNA was extracted for RT-PCR analysis and normalized using cytokeratin-18 expression levels.

Task 1 part 3 of the statement of work was to determine whether hFGFBP1 had a systemic or local effect. The transgenic mice are under a CMV promoter, which is not tissue specific. By transplanting a small fragment of a 6 week old mammary gland from a hFGFBP1 transgenic mouse into a cleared wild type mammary gland, only the mammary tissue would express hFGFBP1 excluding other factors on the mammary development such as the ovaries. Mice were kept on doxycycline diet until transplantation. After hFGFBP1 mammary transplantation at 21 days, mice were left until 120 days of age at which point mammary glands were harvested and examined. Carmine staining of whole mounts showed a significant decrease in tertiary branching similar to what we had observed in the complete transgenic mouse. Therefore hFGFBP1 has a local effect that leads to increased apoptosis and decreased tertiary branching.

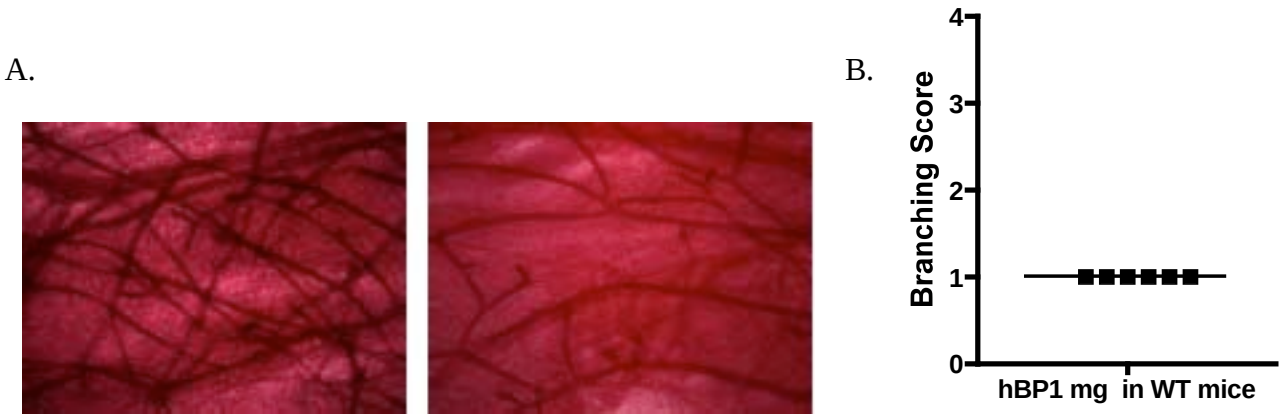

Figure 7. Transplantation of hFGFBP1 transgenic mammary gland tissue into wild type 21 day old cleared mammary fat pads shows a decrease in tertiary branching. A. #4 inguinal mammary glands were harvested at 120 days. Carmine Alum stained whole mounts at 4x showing mammary branching. B. Scoring of 6 transplanted mammary glands into 6 mice. No tertiary branching was observed.

Task 2 from the statement of work was to investigate the effect of hFGFBP1 on mammary gland tumorigenicity. The MMTV-Her2/neu mice are a widely used model that involves a transgene that introduces the rat Erb2 receptor that contains a mutation such that dimerization occurs without binding of ligand similar to what is seen in many human breast cancers. We first determined that mouse FGFBP1 is expressed in normal adult C57/BL6 mouse mammary glands but not in hyperplastic MMTV-Her2/neu mammary tissue (Fig.8). There is some expression of BP1 in Her2/neu mammary tumors but it is lower than normal tissue.

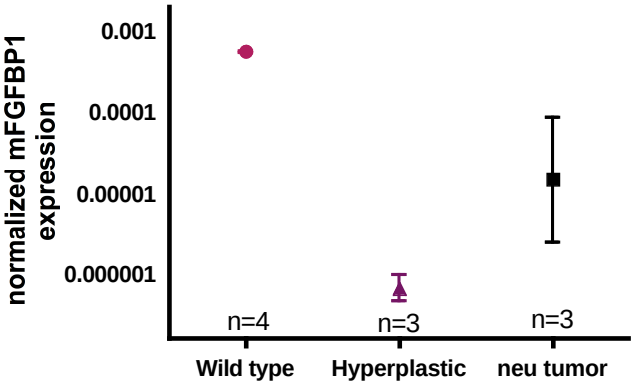

Figure 8. RT-PCR shows decreased expression of mFGFBP1 in HER2/Neu tumors and hyperplastic tissue as compared to wild type littermate levels of mFGFBP1 in normal mammary gland tissue. Samples were normalized by cytokeratin 18 expression levels.

Decreased levels of FGFBP1 in hyperplastic tissue indicated that FGFBP1 might play a role in tumor progression. Our earlier data indicated that FGFBP1 plays a role in apoptosis in mammary gland biology therefore; low expression of FGFBP1 in the HER2/neu model suggested over expression of FGFBP1 may impact normal tumor development. To this end, tet inducible FGFBP1 mice (tTA/tetBP) mice were bred with HER2/Neu mice. However, tumor incidence was dramatically below reported levels (Fig 9) and may be due to strain differences. To determine if this was the case, RT-PCR was used to determine expression levels of the neu transgene (Fig 9).

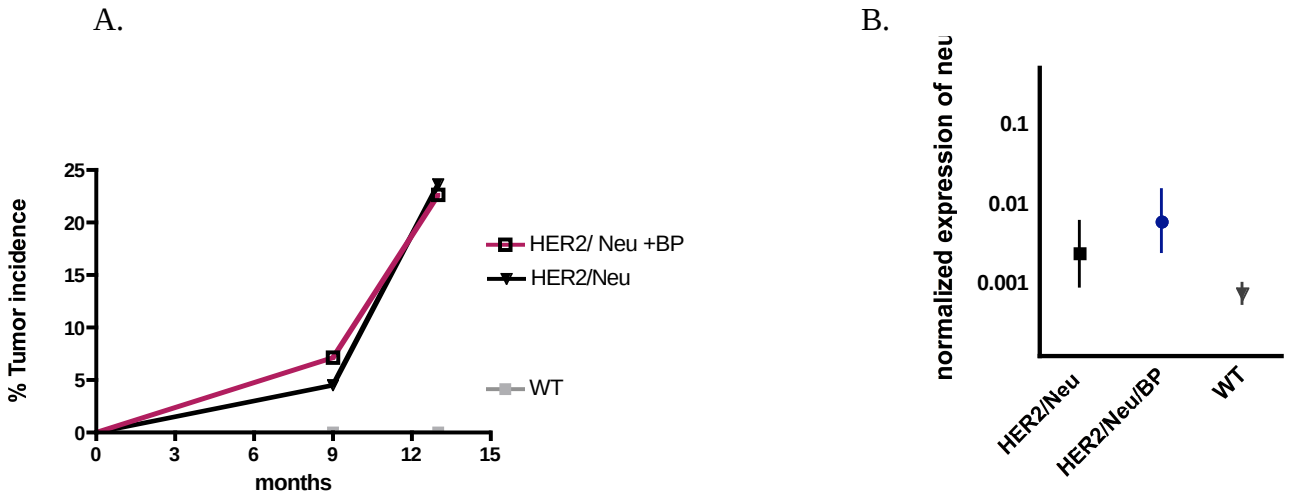

Figure 9.
tTA/tetBP mice were bred with HER2/Neu mice and were monitored for palpable tumors for 15 months. No difference in tumor incidence was seen however, for both HER2/Neu and HER2/Neu/BP mice, the percentage of tumors was much lower than has been previously reported. B. Expression of neu transgene in HER2/neu and HER2/neu/BP mice is low. HER2/neu mice bred with tTA/tetBP mice were induced for 30 days and mammary glands were harvested, processed and evaluated using RT-PCR for neu normalized to actin.

Because neu expression was low and tumor incidence correlated with expression levels, an alternate model was utilized. I trained in Bernd Groner's lab for 3 weeks in 2008 learning to transplant mammary glands, grow primary mammary epithelial cultures and to make lentivirus capable of transducing primary mammary epithelial cells. Figures 10-11 show cloning of hFGFBP1 by PCR from LS174T human colon cancer cells and subsequent insertion into the lentiviral expression pCDH-CMV-MCS-EF1-copGFP vector from System Biosciences. After plasmid was collected, DNA was treated with appropriate restriction enzymes and run on an 1 % agarose gel for visualization. Plasmids were verified by sequencing (McLab, San Francisco) using plasmid specific primers. Both expression plasmid as well as packaging and envelope plasmids were transduced into human embryonic kidney cells (HEK293T) to produce virus. Virus was then administered to HEK293T cells and cells were shown to produce both green fluorescent protein (GFP) and hFGFBP1 at the RNA and protein level (Figure 12).

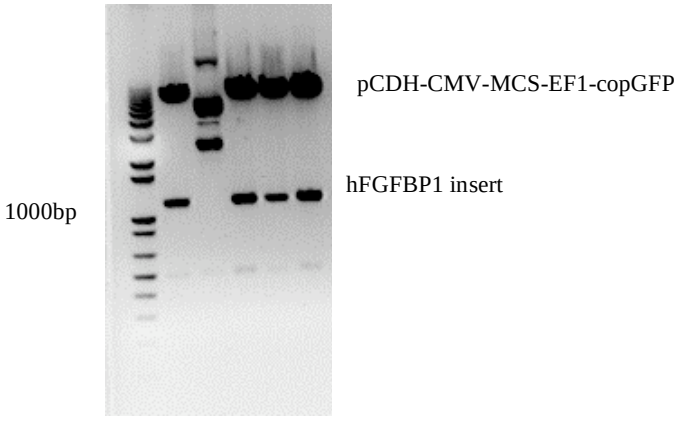

Figure 10. Cloning of hFGFBP1 (hBP1) into pCDH-CMV-MCS-EF1-copGFP vector from System Biosciences with GFP marker. cDNA from LS174T cells was used to PCR the hFGFBP1 insert, which was confirmed by sequencing. After isolation of expression plasmid, restriction enzymes were used to confirm insertion and insert into expression plasmid was confirmed by sequencing using plasmid specific primers.

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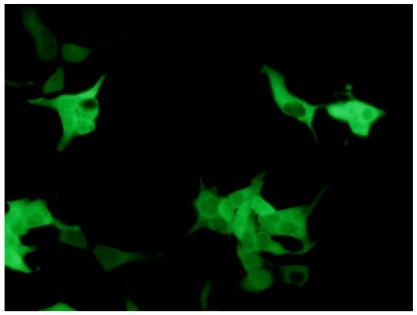

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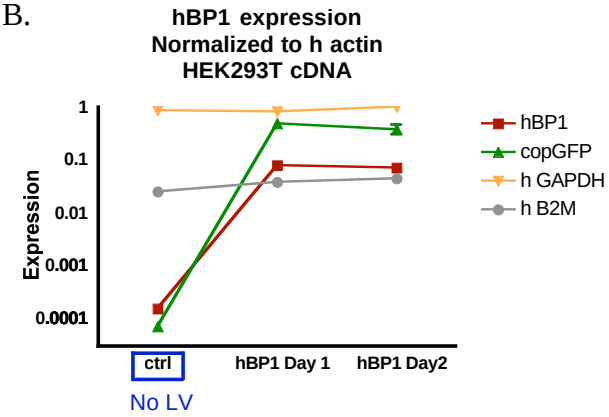

C.

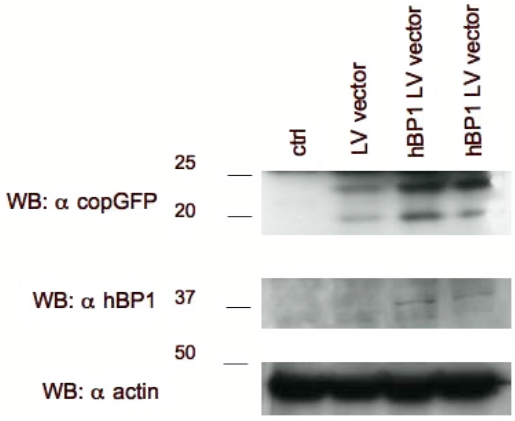

Figure 11. HEK293T human embryonic kidney cells infected with lentivirus express hFGFBP1. A. GFP localization of transduced cells as seen by an inverted stereomicroscope with 400x magnification. B. RT-PCR of HEK293T cells transduced with hFGFBP1 and normalized with actin expression levels. Both GAPDH and B2M were used as control genes. The control cells received no virus (LV). C. Western blot showing both GFP (Evrogen 1:15000) and hFGFBP1 (Monoclonal E311 1:100) proteins are present in transduced cells. Control cells are untransduced HEK293T cells. LV vector is empty copGFP expression vector and hBP1 LV vector contains hFGFBP1 and copGFP.

Spontaneous PyMT tumors were harvested at 1 cm^2 and digested into single cells. These cells were incubated in suspension overnight at 37 degrees Celsius in low adhesion plates to maximize virus to cell surface area ratio as described in (Welm et al. 2008) 2 x 10^5 cells were injected into a cleared fat pad of a wild type 21 day old female. Tumors were monitored and were harvested at 3 weeks post injection. Tumors were measured (Figure

12B) and using the CRI Maestro fluorescence imager, were evaluated for GFP expression to indicate the presence of virally integrated genes either copGFP alone or copGFP + hFGFBP1 (Figure 12A).

A. B.

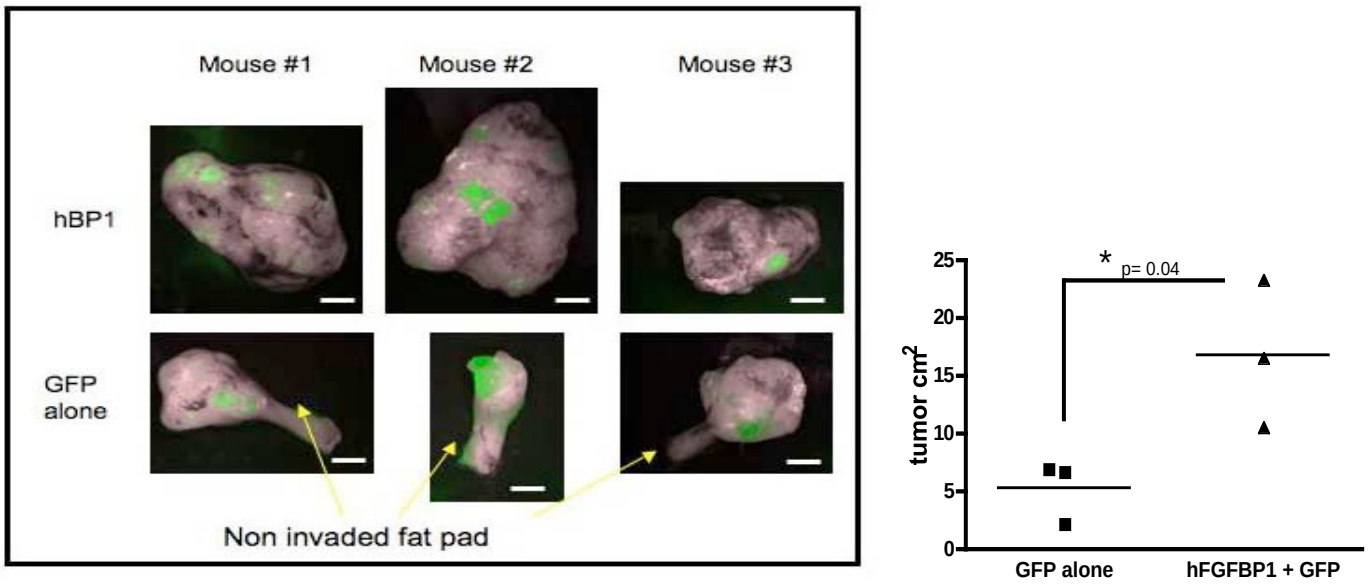

Figure 12. Increased size of tumors with expression of hFGFBP1(hBP1). PyMT tumor cells from a single tumor were dissociated into single cells and incubated with lentivirus containing either GFP alone or GFP + hFGFBP1 overnight. Cells were washed and injected into a cleared fat pad and mice were palpated for tumors daily. The right fat pad of each mouse was injected with hFGFBP1 (hBP1) infected cells and the left with GFP alone. At 3 weeks, tumors were harvested and examined with CRI's Maestro spectral imager to see copGFP (A) and tumors were measured (B).

A significant increase in tumor size was seen in tumors that expressed hFGFBP1.

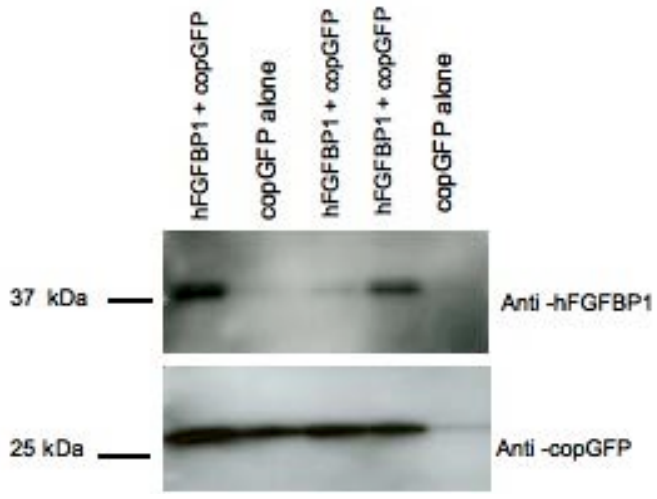

Figure 13. Tumors from Figure 12 were harvested and frozen. Tissue was homogenized in lysis buffer and examined by western blot for expression of copGFP and hFGFBP1 as described above.

KEY RESEARCH ACCOMPLISHMENTS

From Task 1 of Statement of Work

- Figures 1 and 2. Expression of hBP1 transgene in mammary gland in tTA/tetBP transgenic mice
- Figure 3. Decrease in tertiary branching in adult non-pregnant female mice expressing hBP1
- Figure 4. No change in branching development during pubertal mammary developmental stage
- Figure 5. Expression of hFGFBP1 in adult transgenic mice results in increased apoptosis after one week but goes back down to base line levels at 4 weeks.
- Figure 6. mFGFBP1 is highly expressed in adult virgin mammary glands but during times of significant proliferation (prior to lactation) FGFBP1 is down regulated but is again expressed during apoptotic stages (involution).
- Figure 7. Transplant of tetBP/tTA mammary gland into wild type mice shows mammary glands with no tertiary branching.

From Task 2 of Statement of Work

- Figure 8. FGFBP1 is expressed in wild type mammary glands but is not significantly expressed in HER2/neu tumors
- Figure 9. HER2/neu mice crossed with tTA/tetBP transgenic mice show a decreased tumor incidence due to strain decreased neu expression and is not an appropriate model with out further backcrossing.
- Figure 10. hFGFBP1 was successfully cloned into a lentiviral expression system to allow targeted manipulation of primary mammary epithelial cells.
- Figure 11. hFGFBP1 is expressed in HEK293T cells transduced with lentivirus.
- Figure 12. hFGFBP1 expression in PyMT tumors increases tumor size.
- Figure 13. Western blot showing expression of hFGFBP1 and copGFP in transplanted tumors.

REPORTABLE OUTCOMES

1. This work was presented at Georgetown University as a thesis defense entitled " The effects of Fibroblast Growth Factor Binding Protein on embryonic chick and murine mammary development" resulting in the awarding of a doctoral degree in Tumor Biology.
2. Presentation at University of California San Diego on February 22, 2009
3. Presentation at Burnham Institute for Medical Research on March 17, 2009

CONCLUSION

Epithelial branching has long been associated with FGF signaling. FGFs are the most documented mesenchymal factors and while prevalent, overall pathway complexity has left gaps in our understanding of their exact role and implications. Nevertheless, FGF is known to be necessary for the induction of kidney, lung

and salivary gland branching. This is shown by loss of FGF10 or FGFR2 expression in the mouse embryonic lung epithelium, which prevents primary budding and causes organ failure (Peters et al. 1994; Min et al. 1998). Implantation of a bead soaked in FGF10 attracts ectopic branches indicating a role for FGFs in the direction of branching events (Bellusci et al. 1997).

The role of FGF is largely to induce migration and proliferation. However, FGF has been shown to have apoptotic effects in certain instances (Ramos et al. 2006). Specifically, FGF2 induces apoptosis when over expressed in breast cancer cell lines. Furthermore, low levels of FGF2 are associated with a more malignant phenotype in human breast cancer (Luqmani et al. 1992; Lai et al. 1995; Yiangou et al. 1997). Maloof et al. showed that expression of FGF2 decreases Bcl-2 expression in breast cancer cells as opposed to the survival effects seen in fibroblasts, endothelial cells, smooth muscle cells, bladder cancer cells etc (Maloof et al. 1999). The apoptotic effect caused by BP is surprising due to its normal function as an activator of FGF leading to increased proliferation and survival. However, the implications that FGF2 plays a pro-apoptotic role in some settings in breast cancer cells may be reflected in our model. It should also be noted that during progression towards malignancy in mammary tumors, expression of stromal factors FGF2, FGF7 and FGF10 is lost and expression of FGF1, FGF3 and FGF4 is upregulated (Imagawa et al. 2002).

Apoptosis at regular intervals is a normal part of mammary physiology. In murine models, cyclic proliferative activity has been shown with the highest rate observed during late proestrous and estrous. The highest rate of apoptosis is seen during the diestrous phase involving entire alveolar structures (Andres et al. 1995). Although this may account for the increase in caspase-3 positive cells it was seen only in mice that expressed the hBP gene. Later studies should take this into consideration and perhaps monitor cycling to eliminate possible artifacts. Apoptosis during the diestrous phase has been noted largely in the alveolar buds where we saw apoptosis in ductal structures as well as alveolar structures indicating a different effect than that seen during cycling alone. The cyclic regulation of Bcl-2 which inhibits apoptosis, is down regulated during metestrous which occurs immediately prior to diestrous (Andres et al. 1995). Since FGF2 has been shown to alter normal expression of Bcl-2 in breast cell lines, it might explain the increased apoptosis seen in BP induced mammary glands.

Other explanations for the increased apoptosis may be due to up regulation of the inflammatory response by FGF signaling. Welm et al. developed a inducible FGFR1 mammary mouse model that results in increased lateral budding to the point of hyperplasia due to increased proliferation, activation of MAPK and Akt and recruitment of macrophages (Welm et al. 2002). When these mice were crossed with a mouse model that has reduced macrophages, the lateral budding was remarkably reduced. Although this model does not reflect normal FGF signaling due to the receptor design (contains intracellular domain only and uses a Src myristylation sequence to anchor to the membrane), the resulting osteopontin production and macrophage recruitment may be indicative of an FGF response (Schwertfeger et al. 2006). FGFBP has been shown to recruit increased numbers of macrophages during a wound healing study (data not shown) and it may have a similar effect in the mammary gland that would show a similar reduction in lateral budding similar to what is seen in Figure 3.

It should be noted that alterations in FGF signaling in the mammary gland could lead to inappropriate cellular behavior or pathology. In the murine mammary gland, FGF3, FGF4 and FGF8 have been identified as oncogenes after evaluating the effects of proviral insertion of mouse mammary tumor virus (MMTV) (Peters et al. 1983; Peters et al. 1989; MacArthur et al. 1995; Callahan and Smith 2000). Human breast cancer has also shown elevated levels of FGF8 (Marsh et al. 1999) and amplification of FGFR1, FGFR2 and FGFR4 has been identified in breast cancer (Koziczak et al. 2004). While FGFBP has not been shown to interact with all FGFs, it has been shown to bind FGF1, FGF2, FGF4, FGF7, FGF10, and FGF22 among others. Through this modulation, we had expected similar pathologies due to disrupted FGF signaling. The redundancy and further regulation of FGF signaling resulted in altered lateral budding and no malignant phenotypes. However by adding FGFBP1 to PyMT tumor cells, mammary tumor size was increased. **We conclude that although**

FGFBP may act to modulate FGF signaling in murine mammary glands, it is not sufficient to induce a malignant phenotype alone but it does alter normal branching resulting in a phenotype similar to mammary glands without hormonal (progesterone) stimulation. Furthermore, addition of hFGFBP1 to PyMT tumors results in increased tumor size. It is possible that during tumorigenesis, the upregulation (FGF1, FGF3 and FGF4) and down regulation (FGF2, FGF7 and FGF10) of different FGFs may allow FGFBP1 to act in its role as an angiogenic switch by activating those FGFs, while during normal mammary function different FGFs are available leading to increased apoptosis. It may also be at the receptor level where different FGF receptor isoforms activate different signaling pathways and in the tumor setting one FGF receptor may be upregulated (Liang et al. 2008, Elbauomy et al. 2007). Further study of specific FGF signaling and receptor interaction and the role that FGFBP1 may play in regulating apoptosis and vessel growth may give a better understanding of mammary tumorigenesis.

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